Am J Cancer Res 2012;2(3):309-321
www.ajcr.us /ISSN:2156-6976/ajcr0000115

Original Article

Knock down of p53 or its ubiquitin ligase E6AP does not affect the sensitivity of human papillomavirus-positive cervical cancer cells to cisplatin

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Received April 11, 2012; accepted April 20, 2012; Epub April 22, 2012; Published May 15, 2012

Abstract: The persistent infection with high risk human papillomaviruses (hrHPV) is a necessary risk factor for the development of cervical cancer, which is the second most frequent cancer in women worldwide. Cisplatin-based radiotherapy represents the current treatment regimen. However, the results for advanced and recurrent disease are far from optimal. Since almost all cervical cancers contain wild type (wt) p53, which is degraded by the complex of hrHPV E6 and the ubiquitin ligase E6AP, we addressed if the reconstitution of p53 via silencing of E6AP sensitizes cervical cancer cells towards cisplatin treatment. For this we established and characterized two novel cervical cancer cell lines that contain integrated HPV16 genomes. Long-term established HeLa and SiHa cells and the novel cervical cancer cell lines at low passage numbers were treated with different concentrations of cisplatin. Cell viability was measured by the WST-1 assay. In addition, single cisplatin treatment was combined with the silencing of E6AP or p53. The comparison to HeLa and SiHa cells revealed a higher sensitivity of the novel cell lines to cisplatin treatment, which caused p53 accumulation and transcriptional induction of p21. Silencing of E6AP further increased p53 protein levels, but had no effect on cell viability when combined with cisplatin treatment. Interestingly, silencing of p53 had also no effect. We therefore conclude that reactivation of p53 via silencing of E6AP does not increase the sensitivity of cervical cancer cells towards cisplatin treatment.

Keywords: Cervical cancer, HPV, cisplatin, p53, E6AP, chemoresistance

Introduction

Cervical cancer is the second most frequent cancer in women worldwide. It is estimated that approximately 530,000 women developed cervical cancer and that 275,000 died from the disease in 2008 [1]. Thirteen high risk HPV types recognized as class I carcinogens by the WHO are necessary risk factors for the development of cervical cancer and have been implicated in other anogenital cancers and a subset of head and neck carcinoma [1, 2]. Among the hrHPV types, HPV 16 and 18 are found in 70% of all cervical cancer cases [2]. During malignant progression of HPV-induced lesions the HPV DNA is often integrated into the host genome leading to a loss of the E2 and E8^E2C transcription repressors, which might result in an enhanced expression of the viral oncogenes E6 and E7 [3, 4]. Recent publications show, however, that integration of the viral genome can happen already in normal infected tissue and does not occur in a substantial number of cancer cases [5, 6]. HrHPVs encode the potent E6 and E7 oncogenes. E7 causes S-phase entry via disruption of the RB/E2F-family-protein-complex and destabilization of pRB, which would lead to the stabilization of p53 through the activation of p14Arf inhibiting the ubiquitin ligase activity of Hdm-2 towards p53 [7]. To escape p53-triggered apoptosis hrHPVs encode E6 proteins that bind to p53 and to the cellular ubiquitin ligase E6AP (E6-associated protein) which leads to the proteasomal degradation of p53 in HPV-infected cells. Interestingly, the Hdm-2

degradation pathway is completely switched off in HPV-positive cells [8]. As a consequence, pRB and p53 can no longer regulate the G1/S and G2/M checkpoints of the cell cycle and accumulation of chromosomal duplications and centrosomal abnormalities in infected cells takes place [9].

Despite existing screening programs in industrialized countries, an incidence rate of 5-15 per 100,000 cervical cancer cases per year poses a major medical problem [1]. Although two recombinant vaccines against human papillomavirus types 6, 11, 16, and 18, are available since 2006/7 the viral types for which this vaccine provides protection are responsible for only 70% of the cases of cervical cancer. Furthermore, the vaccine will provide no protection for women who have already been exposed to the high risk HPV types. Therefore, the need for advances in the diagnosis and treatment of cervical cancer will remain. Whereas early stages (I-IIa) of cervical cancer can be treated rather successfully, locally advanced disease is characterized by high recurrence rates and a bad prognosis. The standard therapy of locally advanced cervical cancer is a combination of radiation and cisplatin-based chemotherapy with an overall 5-year survival not exceeding 52%, whereas patients with stage IV or recurrent cervical cancer have a median survival time of less than one year [10, 11]. Since the current therapy regimens offer only palliative options for advanced or recurrent disease, there is clearly a need to identify novel approaches that are either more effective, or could be used in combination with current therapy options without increasing side effects. Since almost all cervical cancers contain wild type p53 tumor suppressor protein which is abrogated by degradation through the viral oncoprotein E6, reactivation of p53 seems a promising therapeutic approach. Indeed, it has been shown that restoration of the dormant p53 pathway in cervical cancer cells can inhibit the cell growth [12-15]. Some reports described that interfering with HPV oncogenes E6 and E7 sensitized HPV-positive cells towards cisplatin [16, 17], while other groups described the opposite effects [18]. The conflicting data could be related to differences in the experimental design and to the use of different clones of long-term established cell lines, like HeLa cells. Cell lines cultured in vitro for decades have acquired additional changes and thus, may not be an accurate model for tumor cells in patients with cervical cancer. As cervical cancer can be caused by at least 13 different high risk HPV types, therapeutic strategies aiming at p53 reconstitution should not target the different E6 proteins, which are only modestly conserved in amino acid sequence, but instead address the ubiquitin ligase E6AP as a common target. The goal of this study was to silence E6AP and combine this with cisplatin treatment and test if this combination results in enhanced cell death. The working hypothesis was that restoration of wild type function of p53 could increase the chemosensitivity of cervical cancer cells.

To more closely mimic the in vivo situation, we established two novel HPV16-positive cervical cancer cell lines, CC7 and CC10, and used low passages of those for our analyses. To our knowledge, we describe for the first time the effect of silencing E6AP combined with cisplatin-based treatment in cervical cancer cells. First, we showed that our newly established cervical cancer cell lines, CC7 and CC10, were quite sensitive to cisplatin, whereas HeLa and SiHa cells were much more resistant. Second, we did not observe any changes in cisplatin sensitivity upon silencing of E6AP. Third, silencing of p53 had no effect on the cell viability in combination with cisplatin treatment. Therefore, our results do not support the hypothesis that the restoration of p53 in cervical cancer cells sensitizes these cells for cisplatin treatment.

Materials and methods

Materials

Cisplatin (Cis-diamminedichloroplatinum (II)/ CDDP) was used at 0.5 mg/ml as an isotonic infusion-solution (Medac, Hamburg, Germany). The stock solution was stored at -20°C and was diluted in cell culture medium immediately before use. The following short interfering RNAs (siRNA) were used: siE6AP (human UBE3A [E6AP] L-005137-00, Thermo Fisher Scientific (Dharmacon), Epsom, England); sip53 (tumor protein 53 [TP53] S605, life technologies, Darmstadt, Germany); AllStars Negative Control siRNA (No. 1027281, Qiagen) and CellDeath siRNA (No. 1027299, Qiagen, Hilden, Germany). The following primary and secondary antibodies were used: p53 ([DO-I], Santa Cruz sc-126) and E6AP ([H-182], Santa Cruz sc-25509,

Heidelberg, Germany); alpha tubulin (CP06 Calbiochem, Darmstadt, Germany); polyclonal rabbit anti-mouse immunoglobulin-HRP and polyclonal swine anti-rabbit immunoglobulin-HRP IgG (Dako, Hamburg, Germany).

Cells and cell culture conditions

Cervical cancer biopsies were incubated overnight at 4°C in a dispase II solution (Roche Applied Science, Mannheim, Germany) and then for an additional three hours at 37°C. The biopsies were thoroughly washed in PBS, placed in trypsin solution, cut into small pieces and incubated for 15 min at 37°C. Cells were pelleted by centrifugation, resuspended in fresh E-medium and then seeded on a layer of mitomycinC-treated NIH3T3 J2 feeder cells [19]. Outgrowing cervical cancer cells were split at a ratio of 1:3 after reaching confluency and were maintained in E-medium with feeder cells. The cell lines SiHa (HPV16+), HeLa (HPV18+) and NIH3T3 J2 were cultivated in Dulbecco's modified Eagle's medium (DMEM) (life technologies, Darmstadt, Germany) containing 10% fetal bovine serum (SiHa, HeLa) or 10% calf serum (NIH3T3 J2 feeder) and 50 µg/ml of gentamicin.

HPV genotyping

Genomic DNA was extracted from CC7 and CC10 cells using the EZ1 system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Shortly, cells were harvested and pelleted by centrifugation. The cell pellet was resuspended in EZ1-lysisbuffer containing proteinase K. The digestion was carried out overnight at 55°C while shaking. The genotyping was carried out by INNO-LiPA HPV Genotyping Extra Test System (Innogenetics, Gent, Belgium) [6]. Both cell lines were identified as being positive for HPV16 only.

Quantitative real time PCR (qRT-PCR)

The qRT-PCR was carried out with the Light Cycler® 480 (Roche Applied Science, Mannheim, Germany). All qPCR reactions were run in duplicate and in each run a non-template control was included. To determine the physical state of the HPV16 DNA in CC7 and CC10 cells, the quantification of the HPV16 E2 and E6 genes via qRT-PCR was performed. The integration of the viral DNA into the host genome often results in a loss of the full length E2 expression, thus, leading to an alteration of E2:E6 ratio. Whereas an E2:E6 ratio of 0.0 to 0.2 predicts an integrated DNA state, comparable amounts of E2 and E6 (E2:E6 ratio=0.8-1) could indicate that the viral DNA predominantly exists as episome [6, 20]. qRT-PCR amplification of p21 mRNA was performed using total RNA isolated with the RNeasy minikit (Qiagen, Hilden, Germany) from tissue culture cells and was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The thermal profile and the primers for PGK1 have been described elsewhere [6, 21]. A predesigned primer pair (Hs_CDKN1A_1_SG QuantiTect Primer Assay (200) (QT00062090), Qiagen, Hilden, Germany) was used for detection of p21. The results were analyzed using the LightCycler 480 software, version 1.5 (Roche Applied Science, Mannheim, Germany). Relative expression levels were calculated using the mathematical model described by Pfaffl [22]. To determine the p21 expression after cisplatin treatment or after siRNA transfection, cells were harvested 24h after drug exposure or 48h post transfection, respectively. In all cases, total RNA was isolated from both adherent and detached cells.

Southern blot analysis

Total genomic DNA was isolated by proteinase K and RNAse A digestion in 10mM TRIS pH=7.5, 400mM NaCl, 1mM EDTA. Upon phenol/chloroform extraction, the DNA was ethanol-precipitated. Similar amounts of total DNA were digested with the appropriate restriction enzymes and then separated in a 0.8% agarose gel at 70V for 16h. DNA was then transferred to a positively charged nylon membrane (GeneScreen plus, NEN Dupont, Wilmington, USA) by alkaline transfer. Specific ^{32}P-labeled probes to detect viral fragments were generated with the Ready-to-go DNA labeling kit (Amersham Pharmacia, Freiburg, Germany). Hybridization were carried out in 50% formamide-4XSSPE-5XDenhardt´s solution-1% SDS-20 µg salmon sperm DNA per ml at 42°C overnight (1XSSPE is 0.18M NaCl, 10 mM NaH_2PO4, and 1mM EDTA [pH 7.7]). Blots were washed twice at room temperature in 2XSSC-0.1% SDS, followed by two washes in 0.1XSSC-0.1% SDS and then twice in 0.1XSSC-1% SDS at

50°C (1XSSC is 0.15M NaCl, 0.015M sodium citrate). Hybridizing DNA species were visualized by phosphoimaging on a Fuji BAS reader 1800 (Fuji Photo Film, Düsseldorf, Germany).

Cisplatin-response curves generation and LD values determination

The cells were seeded in triplicate into a 96-well plate using the following cell numbers: $5x10^2$ HeLa cells/well; $1x10^3$ SiHa cells/well; $1x10^3$ CC cells + $5x10^2$ J2 cells/well. 24h after seeding the medium was removed and cisplatin diluted in cell culture medium in a final volume of 100 µl was added. CC cells were exposed to cisplatin concentrations of 0, 1, 2.5, 5, 7.5, 10, 15, and 30 µM. As HeLa and SiHa cells turned out to be more resistant, they were treated with 0, 5, 10, 15, 20, 30, 40, 50, and 100 µM cisplatin for dose-response curves. After 24h drug exposure cell viability was estimated using the WST-1 viability assay (Roche Applied Science, Mannheim, Germany) as described below. Based on the cisplatin-dose response curves we determined the lethal dose values LD30, LD50 and LD70 in respect to the cisplatin treatment. The data were analyzed using GraphPad Prism 5 ® (GraphPad Software, San Diego, USA).

SiRNA treatment and combination with cisplatin treatment

Transfections with siRNA were performed using the HiPerfect reagent (Qiagen, Hilden, Germany) with the fast forward protocol. To investigate the silencing effect of the siRNAs targeting E6AP or p53, 2.5 – $4x10^5$ cells/well were transfected in a 6-well plate with 10 nM siRNA. 24h after transfection the medium was exchanged. 48h post transfection the cells were harvested and the levels of proteins of interest were analyzed by immunoblot analyses. For cell viability assays, the cells were plated at a density of $5x10^3$ cells/well in a 96-well plate. Shortly after seeding the cells were transfected with 10 nM of the respective siRNA. To control for effects of the transfection procedure, the "AllStars" negative control siRNA (Qiagen, Hilden, Germany) was used and all obtained values were normalized to this control which was set to 100% cell viability. To monitor the transfection efficiency a "CellDeath" siRNA (Qiagen, Hilden, Germany) was used as positive control. The cell death induced by "CellDeath" siRNA varied from 64 to 97% depending on the cell line (data not shown). Each experimental design was performed in triplicate. 24h after the transfection the medium was exchanged. In case of the CC cells, $5x10^2$ NIH3T3 J2 fibroblasts per well were added. After another 24h, transfected cells were left untreated (0 µM) or were exposed to different cisplatin concentrations. CC cells were incubated with 5 µM, 10 µM, 15 µM and 30 µM of cisplatin. As HeLa and SiHa cells turned out to be more resistant, they were treated with concentrations of 10 µM, 20 µM, 30 µM and 50 µM. The cell survival was measured by the WST-1 assay 24h after drug treatment 72h post transfection.

WST-1 viability assay

The cell viability was assessed with the cell proliferation reagent WST-1 assay (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Briefly, WST-1 reagent was added to the cell culture and after an incubation for 1h at 37°C, the optical density (O.D.) was measured at 450nm with 650nm as the reference wavelength with a microplate reader (ELISA Processor III Siemens, Eschborn, Germany). The absorbance of the controls was set as 100% viability. The absorbance of treated cells was related and normalized to that of the control-cells. The percentage of surviving cells was defined as: % survival = (O.D. of test sample divided by the O.D. of the control) x 100%.

Immunoblot analyses

Cells including detached ones were harvested by scraping them off in PBS buffer, pelleted by centrifugation, resuspended in 4 x SDS gel loading buffer (Carl Roth, Karlsruhe, Germany) and lysed by boiling for 5 min. NIH3T3 J2 feeder cells were removed from CC cultures one day before harvesting. Total cellular proteins were separated on an 8% SDS-PAGE and transferred in 10 mM N-cyclohexyl-3-amino-propanesulfonic acid (pH 10.3) onto a nitrocellulose membrane (Protran; Whatman, Dassel, Germany). After blocking unspecific binding in Tris-buffered saline (TBS)-0.1% Tween containing 50 mg/ml nonfat dry milk for at least 1h, the blots were incubated with specific primary antibodies at a final dilution of 1:1000. Immunoblot analyses were carried out with mouse or rabbit IgG antibody coupled to horseradish peroxidase at a

final dilution of 1:2000. The antibodies were diluted in TBS-0.1% Tween 50 mg/ml nonfat dry milk. The protein bands were visualized by enhanced chemiluminescence using Super-Signal West Dura reagent (Perbio Science, Bonn, Germany) and a FluorSMax imaging system (Bio-Rad, Munich, Germany). The western blot analyses were performed after 24h cisplatin treatment or 48h after siRNA transfection. The loading of equal amounts of total protein extracts was controlled by detecting alpha tubulin. The quantitative protein analysis was performed with a Quantity One quantification software package (version 4 BioRad, Munich, Germany) with the normalization to alpha tubulin.

Statistical analysis

All data were analyzed using GraphPad Prism 5 ® (GraphPad Software, San Diego, USA). The Mann-Whitney test was performed to test for significant differences in chemosensitivity between different cell lines. *P* values were considered significant if less than 0.05.

Results

Characterization of the novel cervical cancer cell lines CC7 and CC10

All studies investigating chemosensitivity of HPV -infected cells so far used cervical cancer cell lines such as HeLa and SiHa, which have been established decades ago and since then propagated under various tissue culture conditions. Our goal was to study the cytotoxic effects of cisplatin-based chemotherapy combined with a reconstitution of p53 in cell lines that are closer to the tumor cells of cervical cancer patients. Therefore, we established several novel cervical cancer cell lines, designated CC (CC1-CC14), from cervical cancer biopsies. All experiments described here were conducted with the HPV16-positive cell lines CC7 and CC10 at low passage numbers (< 25) and the long-term established cervical cancer cell lines SiHa and HeLa, which contain integrated genomes of HPV16 or HPV18, respectively. First, we determined the physical state of the HPV16 DNA in CC7 and CC10, as the integration state may affect the expression of the viral proteins and therefore, could interfere with the sensitivity to cisplatin. For this, we used quantitative real time PCR

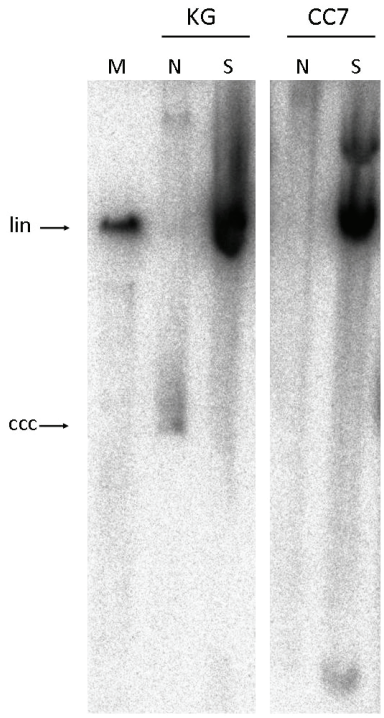

Figure 1. Southern blot analysis of cellular DNA of CC7 cells. Thirty micrograms of cellular DNA was digested with the non-cutting restriction enzyme ApaL1 (N) or with the single-cutter XhoI (S) and separated on a 0.8% agarose gel, blotted, and hybridized to a ^{32}P-labeled HPV-16 full genomic probe. KG cells were used as a positive control for episomal HPV DNA. Lin: linear; ccc: covalent closed circle; M: DNA molecular weight marker.

targeting the E2 and the E6 open reading frames. In CC10, the E2 gene, which is required for viral replication, was nearly absent resulting in an E2:E6 ratio of 0.0. Thus, in CC10 cancer cells the HPV16 DNA is integrated into the host genome. In CC7, an E2:E6 ratio of 0.9 to 1.0 was observed indicating either an episomal state or tandem integrated head-to-tail viral genomes. To differentiate between these possibilities we performed a southern blot analysis with genomic DNA of CC7 cells (**Figure 1**). Extracted DNA from KG cells [19] was used as a positive control for episomal DNA. Southern blot analyzes of total DNA of KG cells resulted in a band migrating typical for covalently closed circular (ccc) HPV DNA. This band was not present in CC7 cells upon incubation with the non-cutter restriction enzyme ApaL1, however, digestion with the single-cut restriction enzyme XhoI revealed a prominent band of the approximate size of the

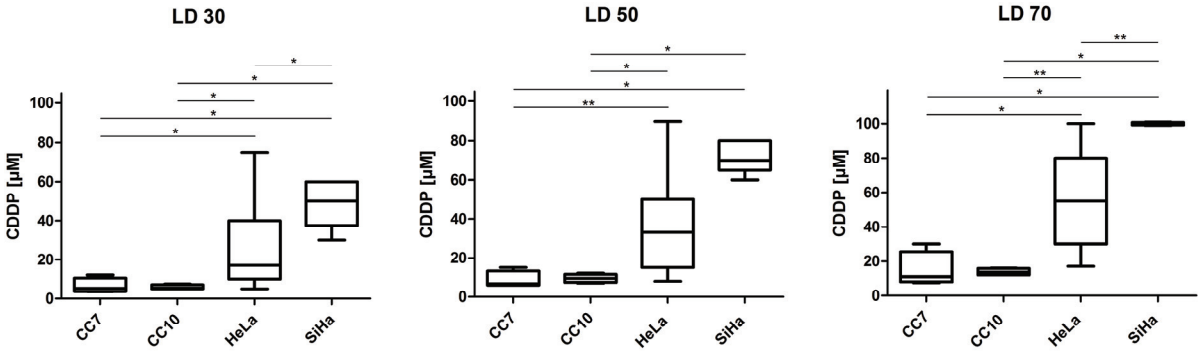

Figure 2. Different responses of cervical cancer cell lines to cisplatin: lethal dose values of CC7, CC10, HeLa and SiHa cells. The cells were treated for 24h with different concentrations of cisplatin (CDDP). Cell viability was determined by the WST-1 assay. The lethal dose values LD30, LD50 and LD70 were determined and plotted in a box plot diagram. The Y-axis represents cisplatin concentration in µM. The lower and the upper quartile correspond to the 25th and 75th percentile, respectively. The box represents the middle 50% including the median shown as a horizontal line. The Mann-Whitney test was used to test for significant differences. *P* values were considered significant if less than 0.05. **P*<0.05; ***P*<0.01; Experiments were repeated for CC7 n=4; CC10 n=4; HeLa n=15; and SiHa n=5. LD30: lethal dose reaching 30% cell death; LD50: lethal dose reaching 50% cell death; LD70: lethal dose reaching 70% cell death.

full-length HPV16 genome and two additional off size bands, which correspond to fragments consisting of viral and cellular DNA at the integration sites. In summary, we established and characterized two novel cervical cancer cell lines, designed CC7 and CC10. Both contain HPV16 DNA which is integrated into the host genome and has lost the E2 gene in case of CC10 cells, whereas in CC7, the viral DNA is probably integrated in head-to-tail tandem repeats.

Cisplatin-response of different cervical cancer cell lines

At present, adjuvant cisplatin-based chemoradiotherapy represents the standard treatment of cervical cancer. Since other publications already showed efficient killing of cervical cancer cells by cisplatin-based monotherapy [23], and clinical trials suggested that under certain conditions neoadjuvant chemotherapy could replace chemoradiotherapy [24] we treated the two novel CC lines CC7 and CC10, as well as HeLa and SiHa cells, with different concentrations of cisplatin to define lethal dose values (LD) 30, 50 and 70 representing 30%, 50% and 70% cell death, respectively. After 24h drug exposure cell viability was determined by the WST-1 assay. All investigated cervical cancer cell lines died after cisplatin treatment in a time- and dose-dependent manner. Based on the survival curves the LD30, LD50 and LD70 values were determined (**Figure 2**). Interestingly, the newly isolated cell lines CC7 and CC10 were significantly more sensitive to cisplatin than the established cell lines HeLa and SiHa. Especially SiHa cells were highly resistant. Whereas in CC cells the LD30 values never exceeded 12 µM, in HeLa and SiHa cells the LD30 values were ~20 µM and ~50 µM, respectively (**Figure 2**, LD30). Furthermore, the cell viability of CC lines was reduced by 50% at ~15 µM cisplatin, whereas for SiHa cells an almost 5-fold higher concentration of cisplatin (60-80 µM) was required to achieve similar cell killing (**Figure 2**, LD50). The same tendency in cisplatin-response was observed for the LD70 values (**Figure 2**, LD70).

Cisplatin-induced effects on p53 tumor suppressor protein

It is generally accepted that the main event responsible for antitumor properties of cisplatin is the induction of DNA damage caused by cisplatin-DNA-adducts [25-27]. DNA damage triggered by cisplatin induces in normal cells a stabilization of the p53 protein via the ATR pathway [28]. Therefore, we first analyzed changes of the intracellular p53 protein levels during cisplatin exposure in cervical cancer cells. The cells were treated with 5 µM, 15 µM and 30 µM cisplatin. After 24h drug exposure all cells were harvested, including detached cells in

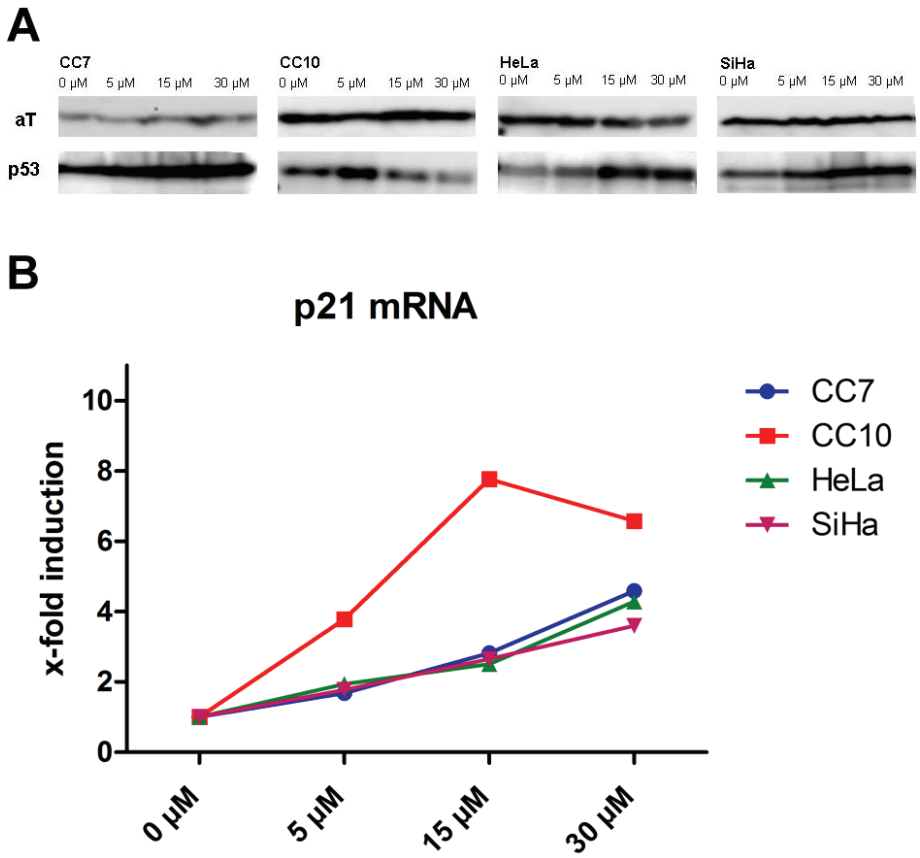

Figure 3. Cisplatin treatment induced p53 accumulation and transcriptional induction of p21. The cells were left untreated (0 μM) or treated with the indicated cisplatin concentrations for 24h. A. Western blot analysis of p53 protein levels upon cisplatin exposure using alpha tubulin (aT) as a loading control. The blot is representative of two independent experiments. B. Expression levels of p21 mRNA were determined by qRT-PCR in untreated and cisplatin-treated cells after 24h drug exposure. The results are shown as x-fold induction relative to untreated cells (0 μM). Values are the mean of two independent experiments.

the supernatant, and western blot analysis for p53 was performed. Cisplatin treatment led to a p53 accumulation in a dose-dependent manner in all cell lines (**Figure 3A**). Only in CC10 cells, p53 protein levels diminished at higher concentrations of cisplatin. Additionally, we determined whether p53 was functionally active and analyzed its transcriptional downstream target p21 at the mRNA level (**Figure 3B**). The p21 expression followed the same kinetics as the cisplatin-induced p53 increase. In CC7, HeLa, and SiHa cells p21 mRNA upregulation was similar ranging between 1.7- and 4.6-fold induction, whereas in CC10 cells, a stronger p21 increase than in other cell lines was observed (3.8 - 7.7-fold).

E6AP as a target to reconstitute p53 wild type function

The transfection with a siRNA against E6AP (siE6AP) silenced E6AP expression in all cell lines tested. The loss of E6AP resulted in p53 accumulation (**Figure 4A**) which was directly linked to a transcriptional induction of p21 (**Figure 4B**). In CC cells an increase of approx. 2.5-fold for p53 and 1.5-fold for p21 was observed. In HeLa cells, we observed the strongest p53 reconstitution (about 60-fold), which correlated with a strong p21 upregulation of 20-fold. In SiHa cells p53 protein levels increased to approx. 2-fold and p21 mRNA to 2.7-fold. To examine whether targeting E6AP expression may interfere with cell viability we performed WST-1 assays 72h post transfection. The obtained cell survival is depicted in **Figure 4C** graph labeled as 0 μM CDDP (first panel). Surprisingly, silencing of E6AP did not affect the cell viability neither in novel nor in long-term established cell lines. In all cell lines the viability of siE6AP-transfected cells was similar to that of control-transfected cells (**Figure 4C**; 0 μM panel).

Combination of siRNA against E6AP and cisplatin treatment had no additive effect on cell killing

We had hypothesized that targeting of E6AP expression could increase the efficacy of

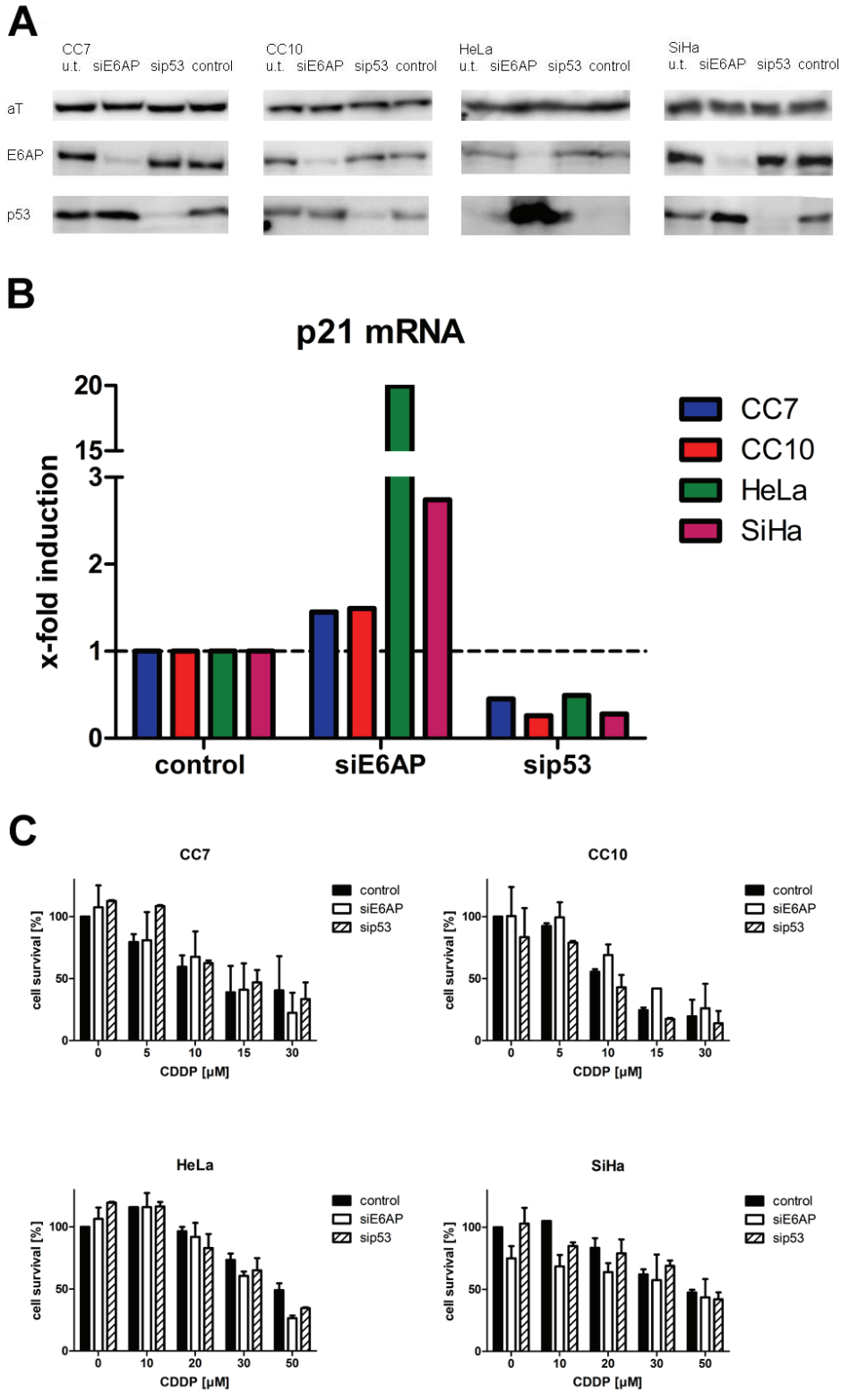

Figure 4. Knock down of E6AP or p53 did not affect the cisplatin-response. A. Western blot analysis of E6AP and p53 protein levels were conducted 48h post transfection. Alpha tubulin (aT) was used as a loading control. Protein levels of p53 upon silencing of E6AP (siE6AP) normalized to control-transfected cells were for CC7: 2.48; CC10: 2.29; HeLa: 57.56 and for SiHa: 1.88. B. qRT-PCR analysis of the expression of p21 mRNA 48h after siRNA transfection shown as x–fold induction relative to control-transfected cells. Values represent the mean of two independent experiments. C. Different cisplatin (CDDP) concentrations were added to the cells 48h post transfection and cell viability was measured after 24h drug treatment. All obtained values were normalized to control-transfected cells, which were set to 100% cell viability. Values represent the mean +/- the standard deviation of two independent experiments. u.t.: untreated; siE6AP: transfected with siRNA against E6AP; sip53: transfected with siRNA against p53; control: transfected with All-Stars Negative siRNA.

cisplatin treatment by reconstitution of p53 wt function, thus, producing a more efficient cell killing upon combined treatment with cisplatin.

To test our hypothesis, the cells were transfected with the siRNA against E6AP (siE6AP) followed by cisplatin treatment. Since

the novel and the long-term established cell lines differ in their cisplatin-response, different cisplatin concentrations for combined treatment were used as before. CC cells were incubated with 5 μM, 10 μM, 15 μM or 30 μM of cisplatin, whereas HeLa and SiHa cells were treated with 10 μM, 20 μM, 30 μM or 50 μM cisplatin. Surprisingly, we did not observe any differences in cell viability between siE6AP-transfected/ cisplatin-treated cells and control-transfected/ cisplatin-treated cells. This outcome was observed in all cell lines studied and at all drug concentrations used (**Figure 4C**).

Silencing of p53 in combination with cisplatin treatment had no additive effect on cell killing

Our observation that there was no additive effect in cell death by reconstituting p53 wt function via the knock down of E6AP in combination with cisplatin led us to further investigate the role of p53 in cisplatin-treated cells. Therefore, we silenced p53 expression using siRNA (sip53) followed by cisplatin treatment. As shown in **Figure 4A**, the transfection with siRNA against p53 reduced p53 expression and this was accompanied by a reduction of p21 mRNA levels of more than 50% in all cell lines tested (**Figure 4B**). However, when p53 depletion was combined with cisplatin treatment, the survival rate of sip53-transfected cells exposed to cisplatin was again comparable to that of the control-transfected cells exposed to cisplatin only (**Figure 4C**). Taken together, there was no additive effect on the cell viability during cisplatin treatment, independent of reconstitution or knock down of p53.

Discussion

Reactivation of the p53 wild type function has been discussed as a promising therapy for cervical cancer. However, the literature published so far revealed conflicting data. Whereas some groups reported inhibition of cell growth without stimulation of apoptosis upon p53 reactivation in HPV related cervical cancer cells [18, 29], other groups described apoptosis as the outcome [12-14, 30]. In addition, induction of senescence upon interference with HPV oncogenes was also described [3, 31, 32]. The use of different strains of long-term established cervical cancer cell lines could be responsible for these conflicting data. Those cell lines have been cultured in tissue culture for decades and contain multiple chromosomal abnormalities and functional defects, and do therefore not represent a good model for tumor cells in cervical cancer patients. In order to overcome this critical issue we established new cervical cancer cell lines, designated CC7 and CC10. Both cell lines contain integrated copies of HPV16 DNA only. To mimic the natural conditions as closely as possible only early passages of these cell lines were used in this study and compared to long-term established cell lines SiHa and HeLa.

We noted significant differences between the newly established CC cells and the long-term established cell lines with regard to the cisplatin sensitivity. For instance, whereas in CC cells the LD30 values never exceeded 12 μM, in HeLa and SiHa cells the LD30 values were ~20 μM and ~50 μM, respectively. Generally, cisplatin-induced DNA damage activates multiple signal transduction pathways including the ATM and/ or ATR protein kinases resulting in phosphorylation of p53 at serine 15 and 20 [33, 34]. These modifications of p53 inhibit the interaction with the ubiquitin ligase Hdm2 leading to p53 stabilization, which now can coordinate repair processes, trigger cell cycle arrest or induce apoptosis. However, in HPV-positive cells the Hdm2 degradation pathway is completely switched off and degradation of p53 is taken over by the ubiquitin ligase E6AP in concert with the viral E6 oncoprotein, which is not affected by N-terminal modification of p53 [8]. However, we observed that cisplatin was able to stabilize p53 in all cell lines investigated indicating that a fraction of p53 in all cells escaped degradation through the E6-E6AP complex. This p53 accumulation was directly linked to transcriptional induction of p21, showing that the cisplatin-induced p53 proteins are transcriptionally active. These findings are in line with other publications where cisplatin-induced p53 stabilization in HPV-infected cells has been observed [35, 36]. Interestingly, the molecular pattern of p53 accumulation and p21 induction was observed at similar time points and already at low cisplatin concentrations, where the difference in cell viability was largest between the novel CC cell lines and the long-term established SiHa and HeLa cells. Therefore, the kinetics of cisplatin induction of p53 and the observed cytotoxicity did not correlate providing a first hint for the

dispensability of p53 in the cisplatin-induced cytotoxicity of cervical cancer cells.

To prove our initial hypothesis of a putative synergism between p53 reconstitution combined with cisplatin treatment we effectively silenced E6AP in all cell lines before cisplatin treatment. The loss of E6AP, as expected, resulted in p53 accumulation which was directly linked to the transcriptional induction of p21. In general, there was already a higher level of p53 protein in untreated CC cell lines in comparison to SiHa and especially HeLa cells. Although we observed differences in the extent of p53 stabilization and p21 induction upon silencing of E6AP there was no correlation between cisplatin-sensitivity and the capability to accumulate p53 upon E6AP loss. Long-term established SiHa and HeLa cells differed greatly in the extent of p53 reactivation and p21 induction indicating a high level of diversity among these cell lines. This observation is supported by earlier findings where RNAi mediated downregulation of E6AP in HeLa, CaSki and SiHa cells revealed only 12 genes commonly affected in all cell lines [37]. Interestingly in the same publication, targeting E6AP expression did not affect the cell viability. Only when the siRNA concentration was increased to 200 nM, an effect towards cell viability was observed 5 to 7 days post transfection. In our study, using lower, but effective concentrations of a siRNA directed against E6AP, we did not observe reduced cell viability in all of the cell lines tested. In contrast, Hengstermann et al. reported growth suppression and stimulation of apoptosis induced by loss of E6AP [38]. The reason for these discrepancies may be explained by the use of different assays to determine cell death. Whereas we used a WST-1 assay measuring viability of the whole cell population, other studies used in situ TUNEL assays and detected apoptosis in single cells, which may lead to an overestimation of the rate of apoptosis induction with respect to the whole cell population. We strongly believe that the results using the WST-1 assay do more appropriately reflect the situation of tumor shrinkage after treatment in the patient situation.

Surprisingly, when we combined E6AP silencing with cisplatin treatment, we did not observe synergistic or additive effects on cell killing, suggesting that reactivation of p53, although it was functional as a transcriptional regulator of the p21 gene, does not play a major role in cisplatin-induced cell death. These results disproved our working hypothesis and the question arose whether p53 in general is required for cisplatin cytotoxicity in cervical cancer cells. Hence, we knocked down p53 using siRNA followed by cisplatin exposure. Again, the cell viability of sip53-transfected cells exposed to cisplatin remained similar to that of the control-transfected cells treated with cisplatin. This outcome demonstrated that p53 might be not necessary for cisplatin-induced cell death and is in contrast to some earlier studies. Koivusalo and coworkers reported that restoration of p53 combined with different chemotherapeutic drugs protected cervical cancer cells from the cytotoxic effects of several compounds but increased the cytotoxicity of others [18]. In the case of cisplatin they reported that activating p53 decreased chemosensitivity. On the contrary, Putral et al. reported that reactivation of p53 via targeting HPV oncogenes increased the sensitivity towards cisplatin in HeLa cells [16]. However, this increased chemosensitivity could not be observed in vivo in a mouse model from the same group [39]. The literature describing the relation between p53 status and sensitivity to anticancer drugs is indeed highly controversial. Whereas in some cancer cells the presence of wild type p53 is reported to render the cells more sensitive [40-42], in other cancers a functional p53 renders the cells more resistant [43, 44]. Moreover, as it is known that p53-negative cells respond to cisplatin, a p53-independent mechanism of action must exist [45, 46]. In agreement with the latter assumption, it has been reported that cisplatin induced the expression of the pro-apoptotic gene Noxa in a p53-independent manner in HeLa cells [47]. The anticancer mechanism of cisplatin is still not fully understood, but it is generally accepted that it acts through cross linking DNA thereby inducing DNA-damage associated apoptosis [27, 48, 49]. However, Mandic et al., showed that cisplatin can also act outside of the nucleus and independent of DNA-damage by inducing ER-stress [50]. Furthermore, cisplatin reacts with many other cellular components such as proteins, cytoskeletal microfilaments and thiol-containing molecules [51-54]. In our analysis, we performed reconstitution of p53 via E6AP silencing or silenced p53 directly in combination

with cisplatin treatment and our results strongly indicate that p53 is dispensable in cervical cancer cells for a successful cisplatin-based chemotherapy.

Acknowledgments

We would like to thank Elke Straub and Angelika Iftner for excellent technical assistance in the establishment of the novel cervical cancer cell lines. This work was supported by the Deutsche Forschungsgemeinschaft SFB773/B4.

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